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A TOXICITY TEST IN ARTIFICIAL SOIL BASED ON THE LIFE-HISTORY STRATEGY OF THE NEMATODE *PLECTUS ACUMINATUS*

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Abstract—The ecological risk assessment of toxicants in soil requires reproducible and relevant test systems using a wide range of species. To supplement present test methods from the Organisation of Economic Cooperation and Development (OECD) in artificial soil with earthworms and springtails, a toxicity test in OECD artificial soil has been developed using the bacterivorous nematode *Plectus acuminatus* (Bastian, 1865) (Nematoda; Plectidae). The juvenile to adult ratio was used as a test parameter since previous life-cycle studies pointed out that fitness of *P. acuminatus* was strongly determined by changes in both reproduction and juvenile survival. Optimal conditions for the performance of nematodes in OECD artificial soil were determined (pH_{KCl} = 5.5, temperature = 20°C, and a moisture content of 70% dry wt. artificial soil), and tests were conducted with cadmium, copper, and pentachlorophenol. After an exposure period of 3 weeks the EC50 for cadmium was 321.0 ± 1.7 mg/kg dry wt., and the no-observed-effect concentration (NOEC) was 32 mg/kg dry wt. The EC50 for pentachlorophenol was 47.9 ± 1.2 mg/kg dry wt., and the NOEC was <10 mg/kg dry wt. For copper the EC50 was 162 ± 0.2 mg/kg dry wt., and the NOEC was 32 mg/kg dry wt. It is concluded that the nematode test may well supplement current soil test systems using earthworms and springtails.

Keywords—Nematodes Toxicity Cadmium Pentachlorophenol Copper

INTRODUCTION

It is generally acknowledged by ecotoxicologists and environmental legislators that single-species toxicity tests provide an adequate first step toward the ecological risk assessment of toxicants in soil and water [1,2]. To obtain safe quality criteria for pollutants in water, many tests have been developed using aquatic species [3], some of which have been standardized and adopted by the Organisation of Economic Cooperation and Development (OECD) [4]. In addition to aquatic tests, progress is made on the development and standardization of soil toxicity tests using a range of invertebrate species [5].

Despite these achievements, which aim for rapid, reproducible, and relevant test systems, little attention is paid to a proper selection of the toxicity parameter based on the life-history strategy of the test species. The life-history strategy reflects the ability of species to withstand adverse ambient conditions and can be represented by changes in life-cycle traits such as survival, reproduction, or longevity. At present, the selection of toxicity parameters is dominated by the assumption that the impact of toxicants on organisms is correctly estimated by measuring the effect on the most sensitive life-cycle traits, such as juvenile survival or reproduction [6–9]. The vulnerability of organisms to chemical stress, however, is not necessarily determined by the effect on susceptible traits. Recent life-history analyses have pointed out that the impact of a chemical depends on the effect on fitness, which is defined by the intrinsic rate of population increase [10]. It was shown that apparently small effects on less sensitive traits may impair the fitness of organisms to a greater extent than large effects on more susceptible traits. Hence, present ecotoxicological tests are not based on considerations from a life-history perspective.

This article describes a soil toxicity test that is based on the life-history strategy of the bacterivorous nematode *Plectus acuminatus* (Bastian, 1865) (Nematoda; Plectidae). It appeared that vulnerability of *P. acuminatus* to toxicants was strongly determined by effects on juvenile survival and reproductive output, not because of their sensitivity to toxicants but because of a strong relationship with fitness [11]. The present toxicity test is based on these traits by means of determining the juvenile to adult ratio.

Bacterivorous nematodes offer perspectives for toxicity testing because a large number of different species can be extracted from the soil efficiently and reared in the laboratory. Moreover, bacterivorous nematodes play a major role in decomposition processes of organic matter by stimulating N-mineralization and nutrient cycling [12]. *Plectus acuminatus* is widely abundant in many soils and has a parthenogenetic life cycle. After a prereproductive period of 3.5 weeks, females produce five to seven eggs per day for a period of 2 months at 20°C (J.E. Kammenga, et al., manuscript in preparation).

Experiments were conducted to determine optimal conditions for population growth and to estimate effect levels at these optimal conditions of cadmium, copper, and pentachlorophenol, which are important environmental contaminants and have different modes of toxic action [13–15]. Previous acute toxicity studies showed that *P. acuminatus* was moderately sensitive to cadmium and pentachlorophenol compared to other nematode species [16].

Toxicity tests were conducted in OECD artificial soil to allow comparison with other tests system using earthworms and springtails.

MATERIALS AND METHODS

Chemicals

Cadmium chloride ($CdCl_2$) and copper chloride ($CuCl_2 \cdot 2H_2O$) were obtained from Merck, Darmstadt, Germany, and pentachlorophenol from Fluka Chemie A.G., Switzerland. The compounds were >99% pure. All other chemicals used (from Merck) were

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of the highest analytical grade. Water was used with a defined mixture of minerals resembling those found in interstitial water of sandy forest soils [17].

Nematodes

Nematodes were originally extracted from the top mineral layer of arable soil at the Binnenhaven in Wageningen, The Netherlands. Stock cultures have been kept in the laboratory for more than 1 year and have subsequently been used for supply of toxicity testing. The cultures of nematodes were kept in agar (0.5%) and fed with *Acinetobacter johnsonion*, a soil-inhabiting bacterium, at a density of 2.10^8 cells/ml. Bacterial densities were measured with a spectrophotometer (Shimadzu, UV-160, 560 nm).

Optimization of test conditions

Preliminary experiments were conducted to determine optimal conditions for population growth of *P. acuminatus* in soil. The influence of soil pH, temperature, and moisture content on population increase was determined. An artificial soil was used [18] which contained (by dry wt.) 70% quartz sand, 20% kaoline clay, and 10% *Sphagnum* peat. Before using the soil for experiments, every batch was heated to 70°C for 2 min using a microwave oven to eradicate unwanted organisms which might be present in one of the soil components.

pH, temperature, moisture content

$CaCO_3$ was used to obtain the following range of pH values, 3.5, 5.5, and 6.5. The pH was measured potentiometrically in a 1-M KCl extract of the soil. The total amount of sand was corrected for the amount of $CaCO_3$ added. Temperature was kept at 20°C, and soil moisture content was 70% dry wt.

Three ambient temperatures were selected, 10, 15, and 20°C ± 0.1°C. The pH was kept at 5.5, and soil moisture content at 70% dry wt.

Three levels of soil moisture content were tested, 50, 60, and 70% dry wt. Temperature and pH were constant at 20°C and 5.5, respectively.

A suspension of *A. johnsonion* was added in all treatments, obtaining a final density of 2.10^9 cells/g dry wt. Artificial soil (5.0 g dry wt.) was inoculated with nematodes (50 adult females, 4 weeks old) which were taken from stock cultures. After 3 weeks nematodes were extracted from the soil by means of a decanting method modified from Cobb [19] (Fig. 1). The number of juveniles and adults was counted in water by using a microscope (magnification ×40–64). Experiments with two replicates were carried out in the dark.

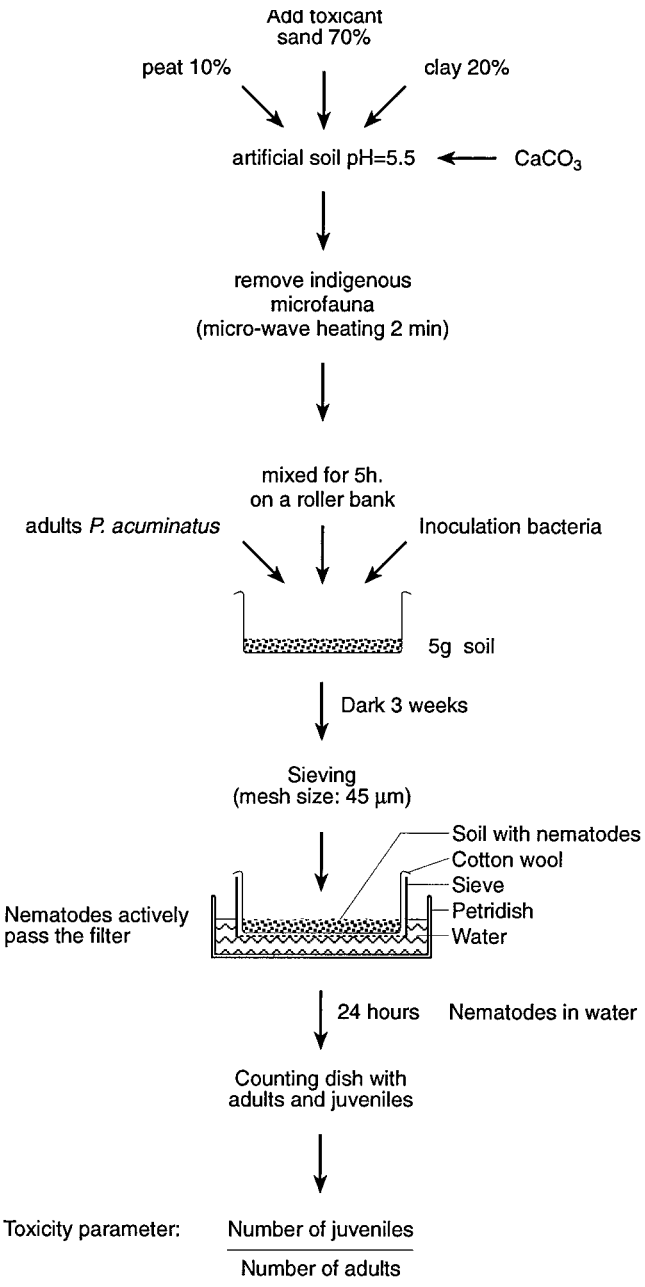

Fig. 1. Experimental design for the extraction of *P. acuminatus* from artificial soil by means of sieving and filtering (for details see Materials and Methods).

Toxicity tests

Cadmium, copper, and pentachlorophenol were mixed with the dry sand fraction before peat and clay were added. The contaminated as well as control soils were homogenized in plastic tubes on a roller bank for 5 h. The following nominal concentration ranges were used: for cadmium (Cd^{2+}) and copper (Cu^{2+}), 0, 10, 32, 100, 320, and 1,000 mg/kg dry wt., and for pentachlorophenol, 0, 10, 18, 32, 56, and 100 mg/kg dry wt. Before using the soil for experiments, every batch was heated using a microwave oven to eradicate unwanted organisms which might be present in one of the soil components.

To obtain a large number of individuals for testing, adult females were allowed to reproduce for 24 h in agar (0.5%) droplets in multiwell dishes (Greiner, 24-compartment plate), after which they were removed. After hatching, juveniles were transferred every third day to fresh agar plates. At the onset of reproduction, 100 nematodes were transferred to 5.0-g dry wt. artificial soil in a Petri dish (6-cm diameter). A suspension of *A. johnsonion* was added to all treatments to obtain a final density of 2.10^9 cells/g dry wt.

Experiments were carried out at optimal conditions in the dark as determined by the optimization experiments, and two replicates were run for each treatment. After 3 weeks the soil in the dishes was washed and sieved (Fig. 1), and the number of juveniles and adults was counted in water by means of a stereomicroscope.

Data analysis

The juvenile to adult ratio per dish was used as a test parameter. The influence of temperature, pH, and soil moisture

Table 1. Influence of pH, temperature (*T*), and moisture content (*M*) on the total number of nematodes per Petri dish and the juvenile to adult ratio (mean and SD in parentheses) in artificial soil ($n = 2$)

	Adults	Juveniles	Juvenile to adult ratio
pH			
3.5	30.5 (2.1)	41.0 (32.5)	1.3 (0.9)
5.5	31.0 (4.2)	313.0 (4.2)	10.2 (1.6)
6.5	22.0 (5.7)	132.0 (25.5)	6.1 (0.4)
T			
10°C	27.5 (13.4)	61.5 (30.4)	2.2 (0.1)
15°C	28.0 (17.0)	103.5 (23.3)	4.2 (1.7)
20°C	36.0 (1.4)	258.0 (25.5)	7.2 (0.4)
M			
50%	33.5 (4.9)	80.0 (9.9)	2.5 (0.6)
60%	39.0 (2.8)	150.0 (45.3)	3.8 (0.9)
70%	22.5 (6.4)	273.5 (17.7)	12.6 (2.8)

content on the ratio was analyzed by analysis of variance. The EC50 values were estimated using nonlinear regression procedures as described in Bruce and Versteeg [20]. No-observed-effect concentration (NOEC) values were determined using Student's *t*-test. All statistical analyses were performed using SAS® software [21].

Chemical analysis

Soil analysis of cadmium and copper was conducted to determine the bioavailability of the compounds. Experiments were carried out separately from the toxicity studies. Artificial soil was contaminated with copper and cadmium using the same procedure as in the toxicity experiments. The soil was extracted with 0.01 M $CaCl_2$ at $t = 0$ (first batch) and $t = 3$ weeks (second batch). Analysis of the extracts was performed with Atomic Absorbtion Spectrophotometry (Perkin Elmer 3030 AAS, flame furnace, detection limit: 0.1 μg/L). Experiments were conducted with two replicates.

Pentachlorophenol concentrations in pore water were obtained from Van Gestel and Ma [22]. Hence, nematodes were exposed to the following range: 0, 0.15, 0.28, 0.53, 0.98, and 1.85 mg/L pore water.

RESULTS

Optimization of test conditions

Table 1 shows the influence of pH, temperature, and soil moisture content on numbers of nematodes and the juvenile to adult ratio. During the experiments, no visible infection of soil by fungi was observed in either treatment.

pH, temperature, moisture content

Total numbers of nematodes were 71, 344, and 154 at pH 3.5, 5.5, and 6.5, respectively. A significant effect of pH on the juvenile to adult ratio ($F = 34.3$, $p = 0.009$), which was highest at pH = 5.5 and lowest at pH = 3.5, was observed.

Temperature had a significant effect on the juvenile to adult ratio ($F = 11.7$, $p = 0.04$). Total number of nematodes was highest at 20°C (294) and lowest at 10°C (89).

Soil moisture content significantly influenced population development of nematodes ($F = 20.4$, $p = 0.01$). Total numbers were highest at 70% dry wt. (269) and lowest at 50% dry wt. (113.5). The juvenile to adult ratio showed the same response. It was observed that a high moisture content decreased total number of adults, perhaps due to decreased food availability. This may lead to a decrease in total juvenile numbers in the long term.

Table 2. Effect of cadmium, pentachlorophenol (PCP), and copper (nominal concentrations) on the total number of nematodes extracted per Petri dish and the juvenile to adult ratio (mean and SD in parentheses)

Nominal concn. (mg/kg dry wt.)	Adults	Juveniles	Juvenile to adult ratio
Cadmium			
0.0	54.0 (5.7)	332.5 (9.2)	6.2 (0.8)
10.0	52.5 (21.9)	447.0 (96.2)	8.9[a] (1.8)
32.0	61.0 (11.3)	340.0 (2.8)	5.7 (1.0)
100.0	56.5 (12.0)	264.0 (73.5)	4.7[a] (0.3)
320.0	66.5 (4.9)	216.5 (106.7)	3.3[a] (1.8)
1,000.0	0.0[a]	0.0[a]	0.0[a]
PCP			
0.0	43.5 (12.0)	274.0 (73.5)	6.3 (0.1)
10.0	60.5[a] (12.0)	240.0 (82.0)	4.9[a] (0.3)
18.0	48.5 (13.4)	378.5 (277.8)	5.3[a] (0.4)
32.0	69.5[a] (4.9)	141.0 (176.8)	1.9[a] (2.4)
56.0	32.5 (45.9)	101.0[a] (130.1)	1.5[a] (2.1)
100.0	0.0[a]	0.0[a]	0.0[a]
Copper			
0.0	54.0 (8.5)	553.0 (26.9)	10.3 (1.1)
10.0	38.0[a] (25.5)	333.0[a] (140.0)	12.9 (12.3)
32.0	39.0[a] (12.8)	355.0[a] (86.2)	9.2 (0.8)
100.0	36.0[a] (22.6)	304.0[a] (147.0)	8.9[a] (1.5)
320.0	60.0 (14.2)	195.0[a] (100.4)	3.1[a] (0.9)
1,000.0	0.0[a]	0.0[a]	0.0[a]

[a]Significantly different from control, $p < 0.05$, $n = 2$.

Toxicity tests

From the optimization studies it appeared that the following conditions were optimal: 20°C, 70% dry wt. moisture content, and $pH_{KCl} = 5.5$. Table 2 shows the numbers of adults and juveniles and the juvenile to adult ratio extracted from cadmium-, copper-, and pentachlorophenol-contaminated soils at these conditions. The ratios were calculated for each replicate.

The total number of adults and juveniles was influenced by the compounds at some concentrations, although there appeared to be no concentration–response relationships except for the number of juveniles in the copper-treated samples.

A concentration–response relationship for the ratio was found for cadmium, pentachlorophenol, and copper, with 100% effect at the highest concentrations (Fig. 2). For cadmium, an increased ratio was found at 10 mg/kg dry wt. The EC50 was 321.0 ± 1.7 mg cadmium/kg dry wt., and the NOEC was 32 mg/kg dry wt. when the positive effects at 10 mg/kg were disregarded. For pentachlorophenol, the EC50 was 47.9 ± 1.2 mg/kg dry wt., and the NOEC was <10 mg/kg dry wt. For copper, an increased ratio was found at 10 mg/kg dry wt. compared to the control. At the other concentrations the ratio decreased. The EC50 was 162 ± 0.2 mg/kg dry wt. The NOEC (32 mg/kg dry wt.) was estimated by disregarding the high ratio at 10 mg/kg.

Total recovery of adults (ratio between number of adults recovered from the soil and the initial number [100] of adults) in the control treatments was 51 ± 6% after 3 weeks.

Chemical analysis

Chemical analysis of exchangeable concentrations revealed that copper was more strongly adsorbed to artificial soil than

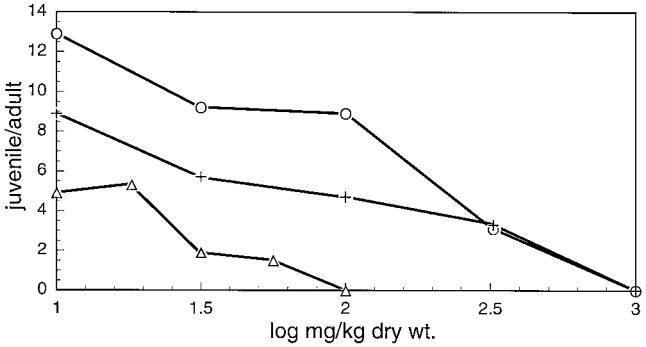

Fig. 2. Dose–response relationship for the juvenile to adult ratio at different nominal concentrations of cadmium (+), pentachlorophenol (△), and copper (○) in artificial soil (±SE).

cadmium (Table 3). At $t = 0$ the exchangeable copper on average for all concentrations was 63 times lower than the total amount added, whereas exchangeable cadmium was only a factor of 12 lower. These results indicate that chemical equilibrium for copper had not yet been reached. It is also shown that copper tends to adsorb more strongly to artificial soil in time than cadmium. After 3 weeks, exchangeable copper was more reduced (factor of 327) compared to cadmium (factor of 17). Hence, it can be concluded that for both cadmium and copper, bioavailability in artificial soil is much lower than the total amount added and moreover is time dependent. It appeared that cadmium and copper were present in the control samples, perhaps because of contamination of one of the soil components (sand, peat, or clay).

DISCUSSION

Life-history strategy

This article presents a toxicity test in which the selection of a suitable effect parameter is based on the knowledge of the life-history strategy of the test organism. Although numerous tests have been developed for a wide range of species, it must be concluded that the selection of effect parameters very often arises from considerations which do not concern life-history theory, such as standardization, ease of recording, cost-effectiveness, rapidity or reliability, and reproducibility [23]. Indeed, a comprehensive life-history analysis aiming to identify key life stages, prior to toxicity testing, is often lacking and difficult to perform.

Table 3. Total amount of copper and cadmium added compared to $CaCl_2$-extracted (0.01 M) fraction in mg/kg dry wt (SD in parentheses) at $t = 0$ and $t = 3$ weeks

	$CaCl_2$-extracted	
Nominal	$t = 0$	$t = 3$ weeks
Copper		
0.0	0.05 (0.005)	<0.05
10.0	0.15 (0.07)	<0.10
32.0	0.45 (0.07)	<0.10
100.0	1.10 (0.07)	0.20 (0.14)
320.0	6.75 (1.91)	0.80 (0.42)
1,000.0	27.90 (29.00)	12.45 (2.33)
Cadmium		
0.0	0.15 (0.70)	0.15 (0.07)
10.0	0.70 (0.14)	0.70 (0.28)
32.0	2.10 (1.20)	0.60 (0.14)
100.0	3.70 (0.70)	6.35 (2.75)
320.0	55.30 (5.37)	55.20 (7.14)
1,000.0	221.35 (11.30)	194.75 (27.22)

Life-history theory provides the tools to gain insight into the relationship between toxicant-induced changes in life-cycle variables and fitness, which is defined as the intrinsic rate of population increase. In general it was found that the vulnerability of species to chemical stress was determined by the relationship beteen life-cycle traits and fitness. For instance, annual iteroparous species, in which the adults survive after reproduction and live on to next year for the following breeding season (e.g., some invertebrates), were vulnerable to stress-induced reduction of adult mortality when the juvenile survival or reproduction is low [10]. Hence, life-history analysis provides the key to identifying important life stages which can be used as suitable and ecologically relevant effect parameters in toxicity tests.

Complete life-history analysis of *P. acuminatus* revealed that changes in juvenile survival and reproduction have a significant influence on fitness [11]. Using a demographic model, it was illustrated that changes in juvenile survival or reproduction and fitness could be described by an exponential curve, thus highlighting the impact of high effect levels in juvenile survival and reproduction on fitness. The present toxicity test combines both these life-cycle traits into one effect parameter, the juvenile to adult ratio.

Nematodes as test organisms

At present, only a few papers have focused on the use of bacterivorous nematodes in toxicity testing. Most of these studies used the same test species, the rhabditid nematode *Caenorhabditis elegans.* The exposure conditions, however, differed too much among the different tests to compare results. Some authors used a special growth medium [24,25] to determine sublethal effects, whereas others used agar as a substrate [26]. Only one paper reported an acute toxicity experiment in soil [27].

Although the biology and genetics of *C. elegans* are well known [28], the use of this species for ecotoxicological testing is rather artificial. All stock cultures used for testing have been kept in the laboratory for decades. In addition, the identification of *C. elegans* and other Rhabditidae extracted from natural soils remains difficult due to inconsistent taxonomic status [29]. These shortcomings may hamper verification of laboratory test results with the outcomes of field experiments. Apart from *C. elegans,* toxicity tests have been conducted using the aquatic species *Panagrellus redivivus* [30,31].

Plectus spp. are bacterivorous and free-living, meaning that they are not associated with plant roots or fungal hyphae. They are abundant in many different soils, and densities may make up more than 50% of the total number of nematodes in the litter layer [32]. Furthermore, many species can be identified since a comprehensive taxonomic revision of the genus [33]. *Plectus acuminatus* can be kept in the laboratory for many generations, and dried cultures can be maintained for several months successfully (unpublished results). Moreover, *P. acuminatus* can be reared in OECD artificial soil, thus enabling a comparison with other standardized test systems using earthworms and springtails [18,34].

Comparison with other tests in artificial soil

The sensitivity of the present test parameter (the juvenile to adult ratio) can be compared with results obtained from other

invertebrate soil toxicity tests using the same artificial soil. We have found an EC50 of 321 mg cadmium/kg dry wt. for the ratio. In one study EC50s for somatic growth and sexual development of 33 to 96 and 108 mg cadmium/kg dry wt., respectively, were found for the earthworm *Eisenia andrei* [35]. For different clones of the springtail *Folsomia candida,* an average EC50 value of 634 mg/kg dry wt. was mentioned for somatic growth [36].

The NOEC of 32 mg/kg dry wt. in the present test was higher than the NOEC for the number of offspring in the earthworm *E. andrei* (10 mg/kg dry wt.) [37] and equal to the NOEC for growth [35]. The ratio in our test appeared to be less sensitive than reproduction in the mite *Platynothrus peltifer* (NOEC = 2.9 mg/kg dry wt.) but more sensitive than reproduction in the springtail *Orchesella cincta* (NOEC = 56.2 mg/kg dry wt.) [38] or *F. candida* (NOEC for population growth = 210 mg/kg dry wt. [39]). Although we compared results among tests performed in the same artificial soil, care must be taken due to differences between exposure routes, either by food (springtails) or pore water (earthworms) and test duration.

For pentachlorophenol, the present test appeared to be equally sensitive (EC50 = 47.9 mg/kg dry wt.) to cocoon production in *E. andrei* (EC50 = 58 mg/kg dry wt.) [40]. When transformed to pore-water concentrations using sorption values [22], EC50 = 0.83 mg/L is comparable to long-term EC50 values obtained for various aquatic organisms [41]. We found an NOEC for pentachlorophenol of <10 mg/kg dry wt., which is comparable to the value found for *E. andrei* on cocoon production [40].

Exposure of *P. acuminatus* to copper resulted in an EC50 of 162 mg/kg dry wt. For *E. andrei* an EC50 of >100 mg/kg dry wt. for growth was found [35]. We found an NOEC of 32 mg/kg dry wt. compared to 56 mg/kg dry wt. for growth in *E. andrei* [35].

The results on exchangeable metal concentrations may have consequences for the estimation of critical effect levels of contaminants. The adsorption of metals to soil components depends largely on the amount of organic material, clay content, and cation exchange capacity [42]. It was shown that adsorption of copper in artificial soil, which contains a relatively large amount of organic matter, was very strong, leading to relatively low available concentrations compared to the total amount added. These findings imply that the effect levels of contaminants on *P. acuminatus,* which are exposed to exchangeable fractions [43], can be compared to similar test systems using other invertebrates in OECD soil.

Explaining variation

It appeared that the values of the juvenile to adult ratio differed among the control treatments. Table 2 shows that they vary from 6.2 to 10.3. The coefficients of variation (C.V.) were 13, 2, and 11% for the control treatments. Relatively high C.V.s were found for the pentachlorophenol treatments (32 and 56 μM), the cadmium treatment (320 μM), and for the copper treatment (10 μM). These large variations may obscure a robust evaluation of the effect assessment on nematodes in OECD soil and hence require special attention.

A major source of variation may be the inhomogeneity in exposure to toxicants which have been added to the soil in the solid phase. To enhance reproducibility we advocate that the toxicants be added as a solution in a smaller amount of soil. This may lead to improved homogenization and hence more uniform exposure conditions

The observed discrepancies might also be attributed to differences in vitality of the stock cultures. Nematode mass breeding cultures prior to toxicity testing require optimal food conditions. This neccesitates regular observation on population viability and food abundance in stock cultures and adequate transfer of populations to fresh food media. The quality of the bacteria may also be very important for growth performance of nematode populations. It is therefore recommended that the rearing of bacteria prior to nematode feeding be standardized.

Furthermore, the inhomogeneity in pH or water-holding capacities (WHC) of the artificial soil may vary among different treatments. We have observed that pH fluctuates during the testing period, which may affect nematode performance and availability of toxicants in the pore water. Small changes in WHC may occur during this period. Despite these large variations, the outcomes indicate the high potential of the method in toxicity testing, and it can be concluded that the present toxicity test using *P. acuminatus* offers perspectives for adequately assessing the effect of contaminants in soil and may well supplement present toxicity tests using other invertebrates in artificial soil. Extensive ring testing, however, should be conducted to determine its reliability and reproducibility among different laboratories.

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